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LONG-TERM FATE OF [^{14}C]NICOTINE IN THE MOUSE: RETENTION IN THE BRONCHI, MELANIN-CONTAINING TISSUES AND URINARY BLADDER WALL

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SUMMARY

N-methyl-^{14}C and 2′-^{14}C-labelled nicotine were used for whole-body autoradiographic distribution studies on C57BL- and NMRI-mice. Radioactivity was retained in the melanin-containing tissues, in the bronchial walls, and in the urinary bladder wall, up to 1 month after administration. The activity levels in the bronchi decreased faster if [2′-^{14}C]nicotine was used. Quantitative measurements of the retention of the 2 ^{14}C-labelled nicotine preparations confirmed the autoradiographic findings.

It is proposed that nicotine is N-demethylated in the bronchial mucosa, the off-coming methyl group being incorporated into the cell constituents of the mucosa. Thin-layer chromatographic studies showed that no nicotine was present in the lungs after 24 h. In melanin, however, only unmetabolized nicotine was found from 4 h on. Some reactive nicotine metabolites may be responsible for the retention in the urinary bladder wall.

Also in the full-term fetuses radioactivity accumulated in the pigmented eyes and in the respiratory tract.

The accumulation and long-term retention of nicotine in the melanin-

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containing structures might accelerate the development of drug-induced or senile changes in these tissues. The retention in the urinary bladder wall persisted even after rinsing. This may indicate an accumulatory mechanism worth considering in the pathogenesis of urinary bladder cancer.

INTRODUCTION

Earlier studies on the distribution of radioactive nicotine in laboratory animals have only involved short exposure times, extending from a few seconds to a couple of hours after administration of the compound [1—4]. In a preliminary study, however, Lindquist and Ullberg [5] have shown that radioactivity can be found in the respiratory tract and in tissues containing melanin up to 30 days after a single intravenous injection of [*N-methyl*-^{14}C]nicotine ([$^{14}CH_3$]nicotine). Moreover, traces of radioactivity could be observed in the urinary bladder wall.

Since only [$^{14}CH_3$]nicotine was used in those whole-body autoradiographic studies, it was unclear whether the radioactivity represented unchanged nicotine, nicotine metabolites or compounds formed after transmethylation or similar 1-carbon transfer reactions. In the present investigation, which involved whole-body autoradiographic studies and thin-layer chromatography of extracts from the eyes and the respiratory tract of pigmented mice, we therefore also used [2′-^{14}C]nicotine, the labelled carbon of which follows the pyridine ring throughout the nicotine metabolism [6].

MATERIALS AND METHODS

Experimental animals

Pigmented mice of the C57BL-strain and albino mice of the NMRI-strain were used in the experiments. The animals were kept on a complete pellet diet (AB Ewos, Södertälje, Sweden) at a room temperature of +25°C, with free access to water.

Compounds

The 2 nicotine preparations were: Nicotine-[*N-methyl*-^{14}C]-*d*-bitartrate with a specific activity of 113 μCi/mg was obtained from the Radiochemical Centre, Amersham, England. Nicotine-[2′-^{14}C]-*d*-bitartrate, spec. act. 20.7 μCi/mg was obtained from Hazleton Laboratories Europe Ltd. The following non-labelled nicotine metabolites were used: Cotinine, hydroxycotinine, and γ-(3-pyridyl)-γ-oxo-*N*-methyl-butyramide were kindly donated by Dr. Herbert McKennis, Jr., Richmond, Va., USA. Nicotine bitartrate was purchased from The British Drug Houses Ltd., England.

Whole-body autoradiography

[$^{14}CH_3$]nicotine was given intravenously to a group of 10 mice, 3 of them

pregnant. The dose was 0.4 μCi/g body wt. to non-pregnant, and 0.25 μCi/g body wt. in the case of pregnant mice. After the injection, the animals were killed by chloroform anesthesia at: 5 min, 20 min, and 1 h (all pregnant); 4 h (1 pigmented and 1 albino); 24 h, 48 h, 4 days, 16 days and 30 days (all pigmented).

Another group of 5 non-pregnant mice was given an intravenous dose of [2′-^{14}C]nicotine (0.13 μCi/g body wt). These animals were killed at 15 min (albino); 4 h, 24 h, 4 days and 32 days after the nicotine administration.

The whole-body autoradiography was performed as described previously [7,8].

Quantitative measurement of the retained radioactivity in the eyes, lungs and the urinary bladder wall

Nicotine, labelled at the 2′- or *N*-methyl position, was injected intravenously to pigmented male mice, divided into 2 groups, each consisting of 16 animals.

The injected doses were 0.4 μCi/g body wt (1.17 μg nicotine/g body wt.) for [$^{14}CH_3$]nicotine (Group I), and 0.074 μCi/g body wt. (1.17 μg/g body wt.) for [2′-^{14}C]nicotine (Group II). 4 animals from each group were killed by cervical dislocation after 20 min, 4 h, 24 h and 4 days survival time. The eyes, the respiratory tract (lungs, trachea and larynx), and the urinary bladder were removed. After the urinary bladder had been opened and thoroughly rinsed with saline solution, the organs were dissolved in 1 ml of Soluene 350® (Packard). When the solubilization was complete, 0.2 ml isopropanol and 0.2 ml conc. H_2O_2 were added and the samples were bleached for 30 min at +40°C. The radioactivity was determined by adding 15 ml Instagel® (Packard) and counting in a Packard Tri-Carb 2425 liquid scintillation spectrometer. External standard was used for correction of the quenching.

Procedure for the isolation and identification of nicotine and its metabolites in pigmented eyes and in the respiratory tract

Extraction. [$^{14}CH_3$]nicotine was injected intravenously to 3 pigmented male mice. The injected dose was 0.4 μCi/g body wt, corresponding to 1.17 μg nicotine/g body wt. The mice were killed by chloroform anesthesia at 20 min, 4 h, and 24 h after the injection. The eyes and the respiratory tract (lungs, trachea and larynx) were removed, homogenized, made alkaline with dilute NH_4OH and extracted with chloroform-methanol (2 : 1 v/v), as described by Hansson et al. [9]. Phase separation was accomplished by centrifugation at 20 000 *g* for 20 min.

Chromatography. The chloroform phase acquired after the extraction of the homogenized pigmented eyes and the respiratory tract with chloroform-methanol (2 : 1 v/v) was subjected to thin-layer chromatography on silica gel according to Hansson et al. [9]. The radioactivity of the chromatograms was detected by autoradiography. The samples were run together with non-radioactive reference compounds (nicotine, cotinine, hydroxycotinine

and γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramid) which were located by spraying the plates with *p*-aminobenzoic acid in 96% ethanol (% w/v) and exposing them to cyanogen bromide vapour.

RESULTS

Whole-body autoradiography

[$^{14}CH_3$]nicotine. A consistently high concentration of radioactivity was found in the melanin-containing tissues and in the respiratory tract following intravenous administration of [$^{14}CH_3$]nicotine in mice. At short survival times there were many sites of localization in addition to these structures, such as the central nervous system, ganglia and the adrenal gland, as previously reported [1,4].

1 h after the injection, the relative concentration in the tissues containing melanin (such as the uveal tract, the skin and the hair follicles) and the respiratory tract became increasingly dominant due to the disappearance of the radioactivity from the other areas. Still 30 days after the injection, the activity of the pigmented uveal tract was very high; significant concentrations were also encountered in the respiratory mucosal lining (nasal, laryngeal, tracheal and bronchial mucosa) (Fig. 1) and in the urinary bladder wall.

In pigmented mice in a late gestation state, a high accumulation was observed in the fetal eye, the fetal inner ear and in the fetal respiratory tract (Fig. 2). The autoradiograms from the adult mice were difficult to interpret with respect to the accumulation in the inner ear because of the difficulty in obtaining good sections through the hard temporal bone.

[2′-^{14}C]nicotine. The distribution of radioactivity after injection of [2′-^{14}C]- or [$^{14}CH_3$]nicotine was very similar at a survival time of 15 min. After 4 h the accumulation of [2′-^{14}C]nicotine in the bronchi was less intense. 4 days after the administration of [2′-^{14}C]nicotine radioactivity remained at high levels only in the melanin-bearing tissues. Also in the urinary bladder wall the activity was relatively high. Detectable amounts were also present in the respiratory mucosal lining (Fig. 3), and in the liver. The retention on melanin and in the bladder wall still persisted 1 month after the administration of [2′-^{14}C]nicotine (Fig. 4).

The [^{14}C]nicotine distribution in the albino mice was equal to that in the pigmented animals with one exception: no accumulation was found in the structures corresponding to the melanin-containing tissues of the pigmented mice at any survival time.

Quantitative comparison of the accumulation of [$^{14}CH_3$]- and [2′-^{14}C] nicotine

The experimental values obtained from liquid scintillation measurements of the radioactivity retained in the lungs, pigmented eyes and the urinary bladder wall at different times after administration of [$^{14}CH_3$] or [2′-^{14}C]-nicotine to mice, are given in Table I. These quantitative data support the autoradiographic findings in showing a much longer retention of the $^{14}CH_3$-

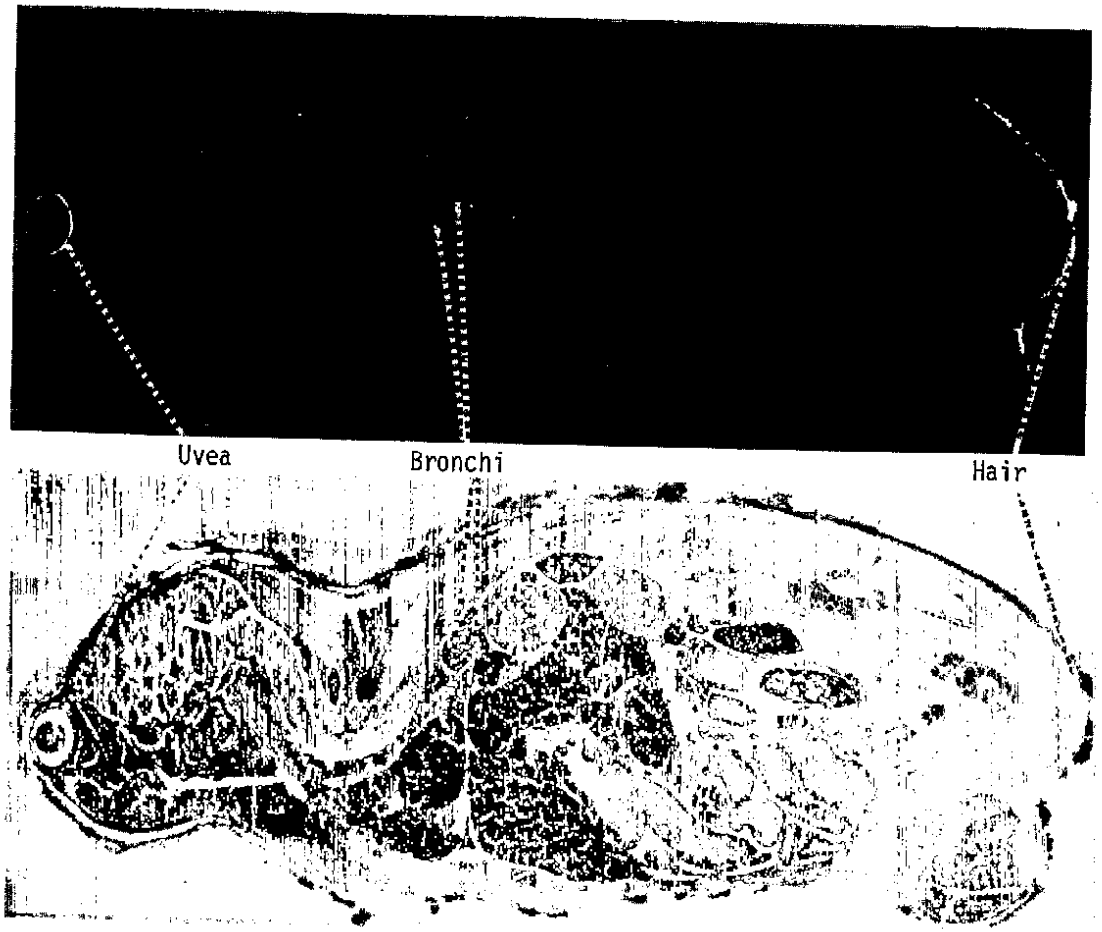

Fig. 1. Autoradiogram (above) and the corresponding section (below) of a pigmented mouse killed 30 days after i.v. injection of [$^{14}CH_3$] nicotine. White areas correspond to radioactivity. There is high retention in the uveal tract, hair follicles and bronchi.

Fig. 2. Details of an autoradiogram of a pregnant pigmented mouse 20 min after i.v. injection of [$^{14}CH_3$]nicotine. There is a high accumulation in the fetal eye. High activity can also be seen in the fetal respiratory tract and intestine.

Bronchi

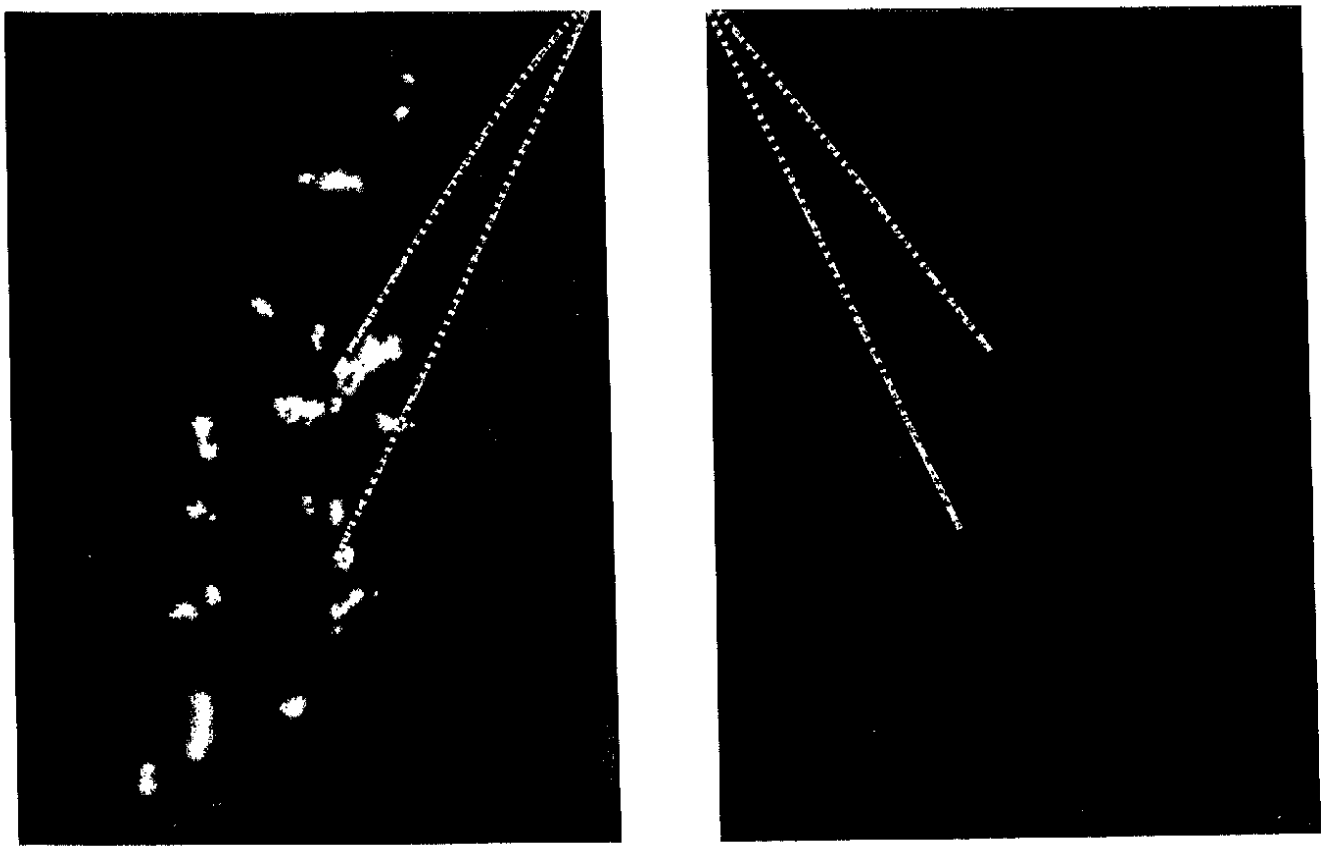

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Fig. 3. Details of autoradiograms showing the lungs of mice killed 4 days after i.v. injection of [$^{14}CH_3$]nicotine (left) or [2'-^{14}C]nicotine (right). There is a marked difference in the amount of radioactivity present in the bronchial walls.

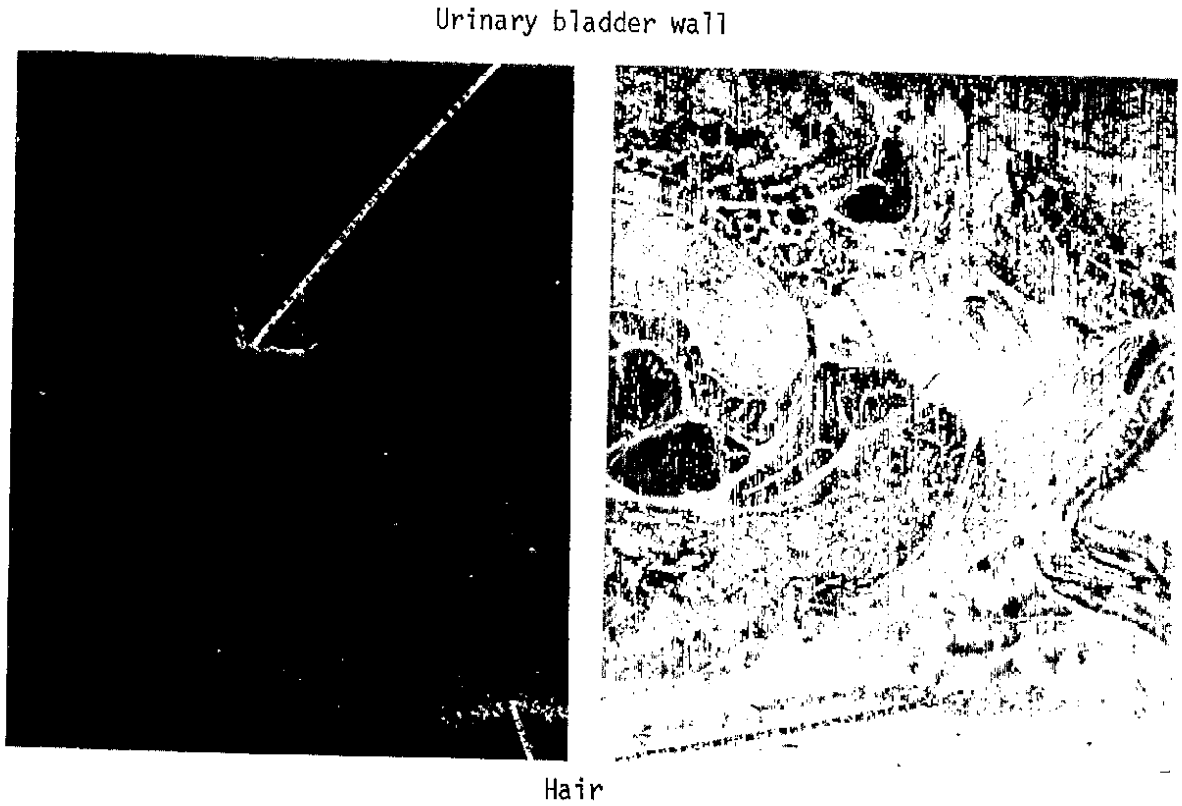

Fig. 4. Detail of an autoradiogram (left) and the corresponding section (right) of a pigmented mouse killed 32 days after i.v. injection of [2′-^{14}C]nicotine. Radioactivity can be seen in the urinary bladder wall and in the hair follicles.

TABLE I

THE AMOUNT OF RADIOACTIVE SUBSTANCE IN THE LUNGS, EYES AND URINARY BLADDER WALL OF PIGMENTED MICE AFTER I.V. INJECTION OF [$^{14}CH_3$]- OR [2'-^{14}C]NICOTINE.

Values are expressed as picomole radioactive substance per organ ±S.D.

Survival time	Lung		Eye		Urinary bladder	
	$^{14}CH_3$	2'-^{14}C	$^{14}CH_3$	2'-^{14}C	$^{14}CH_3$	2'-^{14}C
20 min	817 ± 219	575 ± 87	297 ± 159	299 ± 78	321 ± 190	418 ± 372
4 h	231 ± 24	77.8 ± 9.2	140 ± 12	175 ± 24	37.5 ± 18.9	57.6 ± 48.4
24 h	130 ± 16	19.6 ± 1.9	29.7 ± 2.3	45.0 ± 2.8	7.9 ± 0.5	12.7 ± 6.5
4 d	120 ± 19	7.9 ± 1.3	13.4 ± 2.7	20.1 ± 3.1	7.9 ± 3.0	1.2 ± 0.4

label in the lungs. After 4 days, the radioactivity levels were 15 times higher if [$^{14}CH_3$]nicotine was injected than if [2'-^{14}C]nicotine was administered.

In the pigmented eyes and the urinary bladder wall, the differences in accumulation between the 2 labellings of nicotine were not very dramatic, although the [$^{14}CH_3$]nicotine values were generally somewhat lower. The only exception was the bladder wall at 4 days' survival time, where [$^{14}CH_3$]-nicotine gave a higher activity level.

Thin-layer chromatography of extracts from pigmented eyes and respiratory organs

Thin-layer chromatography was performed on the chloroform phases acquired by extraction of the eyes and the respiratory tract after administration of [$^{14}CH_3$]nicotine to pigmented mice.

A radiochromatogram of the chloroform phases of the extracts from the eyes removed at 20 min, 4 h and 24 h after the injection of [$^{14}CH_3$]nicotine is shown in Fig. 5. The extract obtained 20 min after injection exhibited 3 spots which had the same R_F-values as the reference substances nicotine (R_F = 0.72), cotinine (R_F = 0.47)and γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide (R_F = 0.33). In contrast, the extracts obtained 4 h and 24 h after injection gave 1 spot only which had the same R_F-value as nicotine.

A radiochromatogram of the chloroform phases from the extracts of the

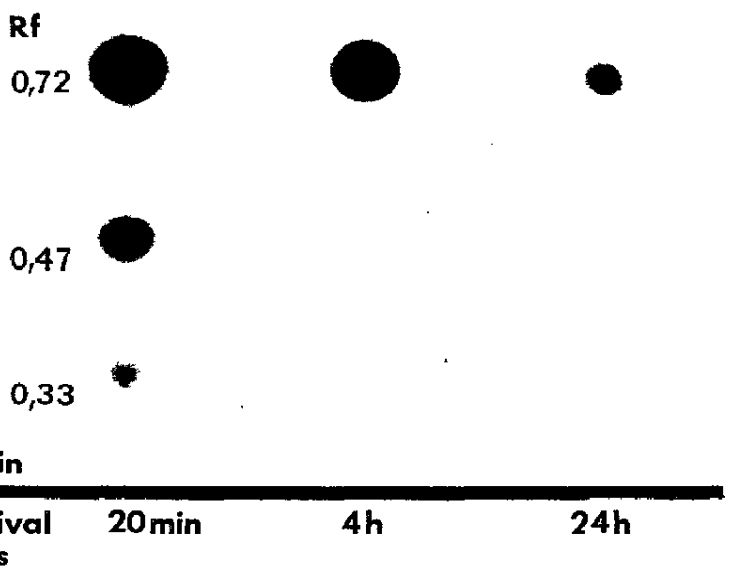

Fig. 5. Radiochromatogram of chloroform extracts from the eyes of pigmented mice 20 min, 4 h and 24 h after i.v. injection of [$^{14}CH_3$]nicotine. Solvent system: ethanol—acetone—benzene—conc. NH_4OH (5 : 40 : 50 : 5). Components: nicotine (R_F = 0.72), cotinine (R_F = 0.47), γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide (R_F = 0.33)

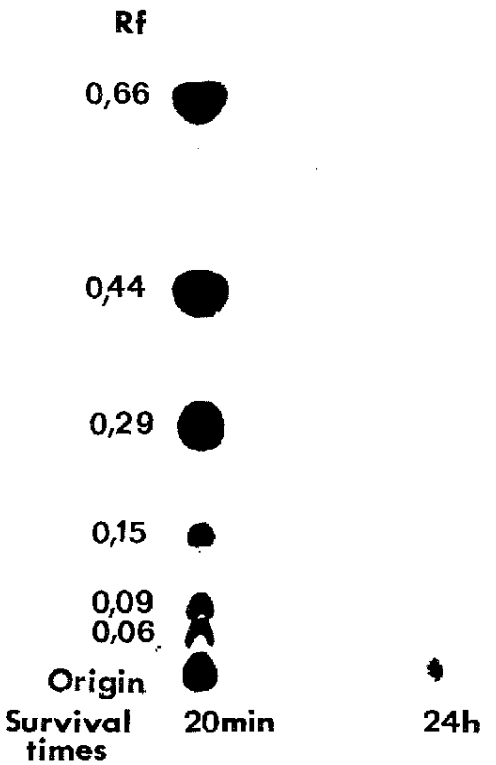

Fig. 6. Radiochromatogram of chloroform extracts from the respiratory tract (lungs, trachea, larynx) of mice 20 min and 24 h after i.v. injection of [$^{14}CH_3$]nicotine. Solvent system: ethanol—acetone—benzene—conc. NH_4OH (5 : 40 : 50 : 5). Components: nicotine (R_F = 0.66), cotinine (R_F = 0.44), γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide (R_F = 0.29), hydroxycotinine (R_F = 0.15). R_F = 0.09 and R_F = 0.06 were not identified.

respiratory tract (lungs, trachea and larynx) removed 20 min and 24 h after the injection of [$^{14}CH_3$]nicotine is shown in Fig. 6. The extract obtained 20 min after the injection showed 7 spots, 4 of which had the same R_F-values as nicotine (R_F = 0.66), cotinine (R_F = 0.44), γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide (R_F = 0.29), and hydroxycotinine (R_F = 0.15). The extract obtained 24 h after the injection gave only 1 radioactive spot which was close to the origin. Preliminary chromatographic studies of extracts from the respiratory tract using different solvent systems indicate that the "origin activity" consists of compounds having an amino acid character.

DISCUSSION

Lungs

Nicotine accumulated rapidly in the bronchial walls after injection, as

shown by the autoradiograms, whether [2′-^{14}C]- or [$^{14}CH_3$]nicotine was used (see also Wadell and Marlowe [4]). The level of radioactivity in the bronchi decreased faster in the case of [2′-^{14}C]nicotine than in the case of [$^{14}CH_3$]-nicotine. No unmetabolized, extractable nicotine was found in the lungs 24 h after an intravenous injection of [$^{14}CH_3$]nicotine. Therefore it may be concluded that *N*-demethylated nicotine metabolites are formed. In the lung, the *N*-demethylation is likely to occur in the enzymatically very active bronchial mucosa [10] where the off-coming methyl group could be incorporated into the 1-carbon pool.

Turner et al. [11] have found that demethyl cotinine can be formed from nicotine in the isolated dog lung, and Oppelt et al. [12] and Litterst et al. [13] have demonstrated the capacity of lung preparations from various species to *N*-demethylate drugs. Furthermore, it is known that 10—15% of the dose of [$^{14}CH_3$]nicotine given to mice is excreted as $^{14}CO_2$ within 24 h [1,14].

Whether a strong accumulation and concomitant metabolism of nicotine in the bronchial mucosa has any toxicological implication or not is uncertain. It is difficult to estimate what the incorporation of a methyl group originating from nicotine in the cell components of the bronchial mucosa may imply. However, there are presently no reasons to believe that it has any great toxicological significance. Of greater importance is probably the observation that [2′-^{14}C]nicotine even 4 days after a single injection gives rise to a low but, on the autoradiograms, still clearly visible amount of radioactivity in the bronchial walls (Fig. 3). A possible explanation for this is that the fairly stable nicotine iminium ion intermediate, postulated by Nguyen et al. [15] to appear during *N*-demethylation of nicotine in rabbit liver homogenates, could also occur in vivo and interact with nucleophilic components at the site of formation to give products that are retained.

Melanin-containing tissues

The melanin-containing tissues exhibited — as shown autoradiographically — high levels of radioactivity at all survival times irrespective of whether [2′-^{14}C]- or [$^{14}CH_3$]nicotine was employed. It seems likely therefore that the radioactivity represents mainly unaltered nicotine. This is corroborated by the chromatographic result (Fig. 5) which shows nicotine to be the only labelled compound in the extracts from the pigmented eyes 4 h or more after administration of nicotine.

Since no accumulation of [^{14}C]nicotine could be found in the eyes of the albino mice, it is evident that it is the melanin in the pigmented eyes that binds the drug. Furthermore, synthetic melanin has a high binding capacity for nicotine in vitro, but a low affinity for cotinine, the main nicotine metabolite (unpublished results).

Drug-induced lesions in tissues containing melanin, such as the eye, the inner ear and the brain stem, have been related to a melanin affinity [16—18]. From available data on absorption of nicotine from cigarettes [19] it can be calculated that certain heavy smokers may receive daily doses of

nicotine exceeding 100 mg. It cannot be excluded that the regular intake of nicotine in this dose-range may induce the same type of lesions as may occur after long-term medication with drugs having melanin affinity. It is also possible that nicotine and other chemicals with a melanin affinity may have an additive effect, resulting in toxic lesions in tissues with melanin. Fetal melanin-containing tissues may be more sensitive to drugs than the corresponding adult structures. Furthermore, senile degenerative changes in the melanin structures may be accelerated by the action of nicotine.

Urinary bladder wall

The finding of a retention in the urinary bladder wall indicates that some nicotinic components have been accumulated in the bladder cells. As radioactivity persisted after rinsing of the bladders, some of these nicotinic components are apparently firmly bound to the cell constituents. The uptake may have taken place through the blood vessel walls or perhaps more likely by diffusion from the urine.

The accumulation of potentially harmful substances in the cells of the urinary bladder wall may be an intermediate step in the development of cancer and other chronic ailments at this site. The possible role of nicotine metabolites in this connection is unknown.

Heavy smoking has been related to an increased occurrence of urinary bladder cancer [20–22]. The component of tobacco smoke responsible for this has not yet been determined.

Fetal distribution

The whole-body autoradiograms from pigmented C57BL-mice in a late gestation stage showed that a radioactive substance passed the placenta and accumulated in the pigmented fetal eyes, in the intestine, and in the respiratory tract after administration of [$^{14}CH_3$]nicotine (Fig. 2). Similar results have been reported by Tjälve et al. [20] who used unpigmented NMRI-mice in their investigation. In this case, no accumulation was observed in the fetal eyes.

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